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Evaluation of Water-Soluble Pneumocandin Analogs L-733560, L-705589, and L-731373 with Mouse Models of Disseminated Aspergillosis, Candidiasis, and Cryptococcosis

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The activities of the water-soluble pneumocandin derivatives L-733560, L-705589, and L-731373 were evaluated in mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis and were compared with those of commercially available antifungal agents. Pneumocandins are inhibitors of 1,3-β-D-glucan synthesis. In the aspergillosis model, L-733560 and L-705589 significantly prolonged the survival of DBA/2N mice challenged intravenously with *Aspergillus fumigatus* conidia. L-733560 and L-705589 exhibited efficacies comparable to that of amphotericin B (AMB) with 90% effective doses of 0.48, 0.12, and 0.36 mg/kg of body weight, respectively. Two mouse models of disseminated candidiasis were used to evaluate these compounds. In both models, mice were challenged intravenously with *Candida albicans*. In a *C. albicans* survival model with DBA/2N and CD-1 mice, the efficacy of L-733560 was comparable to that of AMB, while L-731373 and L-705589 were somewhat less active. In a previously described *C. albicans* target organ kidney assay, the pneumocandin analogs and AMB at doses of ≥0.09 mg/kg were effective in sterilizing kidneys, while fluconazole and ketoconazole were considerably less active and did not sterilize kidneys when they were used at concentrations of ≤100 mg/kg. Although orally administered L-733560 showed activity in both candidiasis models, its efficacy was reduced compared with that of parenterally administered drug. In a disseminated cryptococcosis mouse model that measures the number of CFU of *Cryptococcus neoformans* per gram of brain and spleen, L-733560 at 10 mg/kg was ineffective in reducing the counts in organs, while AMB at 0.31 mg/kg sterilized the organs. These results indicate that the pneumocandins may be beneficial as potent parenterally administered therapeutic agents for disseminated aspergillosis and candidiasis.

The expanding population of immunocompromised patients seen over the last decade has resulted in an increased incidence of opportunistic mycoses. Immune deficiencies resulting from AIDS, organ transplants, immunosuppressant chemotherapy, and other iatrogenic or nosocomial situations have predisposed an increasing segment of the population to these infections and have created a critical need for new, safe, fungicidal agents that can be used to treat these life-threatening, disseminated diseases. Since diagnosis of suspected fungal infections is often unpredictive or delayed because of the nature of the diagnostic techniques that are used, current therapy is frequently initiated empirically and still relies on amphotericin B (AMB) as the drug of choice because of its broad-spectrum and fungicidal activity. However, AMB is relatively toxic, and its clinical utility is limited to controlled intravenous (i.v.) administration and thus is used primarily to treat deep-seated fungal infections (13, 21). The newer azole antifungal agents have broad spectra of activity, are orally active, and are considered to be less toxic than AMB but are fungistatic against most major fungal pathogens (21).

The pneumocandins (lipopeptides) are cyclic hexapeptides with fatty acyl side chains similar in structure to the echinocandin class of antifungal agents (2, 8). Previously reported pneumocandins have been shown to be fungicidal in vitro and to have an antifungal spectrum limited mainly to *Candida* species (3, 4). In vivo efficacy has been reported in animal models of disseminated candidiasis (4, 5), oropharyngeal and gastrointestinal candidiases (12), and *Pneumocystis carinii* pneumonia (3, 26, 27). The term "pneumocandin" has been coined from the anti-*P. carinii* and anti-*Candida* activities that these compounds possess (13, 28). The pneumocandins are inhibitors of 1,3-β-D-glucan synthesis, a critical structural cell wall component in certain pathogenic fungi and *P. carinii* cysts (2, 4, 8, 19, 20). In the study described in this report, the in vivo activities of the water-soluble pneumocandin derivatives L-733560, L-731373, and L-705589 were evaluated in mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis.

MATERIALS AND METHODS

Drugs. The pneumocandins L-733560, L-705589, and L-731373 were synthesized by the Department of Synthetic Chemical Research at Merck Research Laboratories, Rahway, N.J. The pneumocandins and fluconazole (FCZ; Pfizer, Groton, Conn.) were formulated in sterile distilled water. Itraconazole (ITZ), which was received from Janssen (Beerse, Belgium), was suspended and diluted in aqueous vehicle (AV) solution (Merck & Co., West Point, Pa.). AV solution consists of 0.9% sodium chloride, 0.5% carboxymethyl cellulose, 0.4% Tween 80, and 0.9% benzyl alcohol. Ketoconazole (KTZ; Janssen) was solubilized in 0.2 N HCl. AMB was purchased as Fungizone (E. R. Squibb & Sons, Princeton, N.J.) and was reconstituted according to the manufacturer's instructions and diluted in sterile water.

Animals. Outbred, female CD-1 mice (average weight, 19 to 21 g; Charles River, Wilmington, Mass.) were used in the disseminated candidiasis survival studies. Complement component C′5-deficient DBA/2N female mice (average weight, 19 to 21 g; Taconic Farms, Germantown, N.Y.) were used in the disseminated aspergillosis and candidiasis survival studies and in the *Candida* and *Cryptococcus* target organ assays because they have previously been shown to be highly susceptible to fungal infections (16).

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All procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were approved by the Merck Institutional Animal Care and Use Committee. Procedures for the care and use of research animals at Merck meet or exceed all applicable local, national, and international laws and regulations.

Organism and culture conditions. *Aspergillus fumigatus* MF 5668 (ATCC 13073), originally isolated from a human pulmonary lesion, was cultured on Sabouraud dextrose agar (SDA; BBL, Cockeysville, Md.) slants at 30°C for 4 to 5 days. Conidia were washed from the surfaces of several (three or four) agar slants into sterile saline with 0.01% Tween 20 (Fisher Scientific, Fair Lawn, N.J.), and the conidium concentration was quantitated by counting with a hemacytometer. The viable count was confirmed by serially diluting the conidial suspension 10-fold and plating the inoculum onto SDA plates. Merck cultures of *Candida albicans* MY1055 (a human isolate from Williamsburg Community Hospital, Williamsburg, Va.) and *Cryptococcus neoformans* MY2061 (a human isolate obtained from the University of Wisconsin, Madison) were grown on SDA plates at 35°C for 24 and 48 to 72 h, respectively. Yeast cells were washed from the surfaces of one to two SDA plates, and cell concentrations were quantitated by counting with a hemacytometer. The viable count was confirmed by serially diluting the cell suspension 10-fold and plating the inoculum onto SDA plates.

Survival studies. Disseminated aspergillosis was induced in DBA/2N mice by the i.v. inoculation of 0.2 ml of the spore suspension (5.0×10^5 to 1.0×10^6 conidia per mouse) into the lateral tail vein. The inoculum of *A. fumigatus* MF 5668 was designed to deliver approximately three to four times its 14-day 50% lethal dose, which resulted in mice showing the first signs of infection by days 3 to 5, the first deaths occurring by days 5 to 7, and 90 to 100% mortality occurring by day 21 after challenge.

Disseminated candidiasis was induced in DBA/2N and CD-1 mice by the i.v. inoculation of 0.2 ml of a *C. albicans* MY1055 cell suspension into their lateral tail veins. DBA/2N mice received 10^6 blastoconidia per mouse, and CD-1 mice received 10^7 blastoconidia per mouse; these inocula were previously determined to represent one 14-day 100% lethal dose for each strain of mouse.

Therapy was initiated within 15 to 30 min after challenge, and mice were treated for a total of 4 days in the candidiasis model and 5 days in the aspergillosis model. The pneumocandins were administered intraperitoneally (i.p.) twice daily (b.i.d.). L-733560 was also tested orally (p.o.) b.i.d. in the disseminated candidiasis models. AMB was administered i.p. once daily (q.d.). FCZ, KTZ, and ITZ were given p.o. q.d. Compounds were tested at titrated concentrations (serial fourfold dilutions), with 10 mice per therapy group. Infected sham-treated mice were administered sterile water. Morbidity and mortality were recorded daily for 28 days.

Target organ studies. DBA/2N mice were infected i.v. with approximately one 50% lethal dose of *C. albicans* MY1055 (7.5×10^4 blastocondia per mouse) or *C. neoformans* MY2061 (1×10^6 cells per mouse). Therapy was initiated within 15 to 30 min after challenge, and mice were treated for a total of 4 days. Compounds were administered as described above. The target organ assay for *C. albicans* monitors the number of CFU per gram of paired kidneys at time points following challenge (target organ kidney assay [TOKA]). The target organ assay for *C. neoformans* monitors the number of CFU per gram of brain and spleen at time points following challenge (target organ brain and spleen assay). Organs from sacrificed mice (five mice per group per experiment) were removed by using aseptic technique, weighed, and placed in sterile Whirl Pak bags (Fisher Scientific) containing 5 ml of sterile saline. The organs were homogenized in the bags and were serially diluted in saline, and aliquots were plated onto SDA plates. The plates were incubated at 35°C, and the organisms were enumerated after 48 h for *Candida* spp. and 72 h for *Cryptococcus* spp. The mean number of CFU per gram of tissue in the organs of the treated groups was compared with those in the organs from sham-treated control animals. Percent clearance indicates the number of mice in which there were no detectable yeasts and in which the limit of detection, because of the dilution scheme, was 50 yeast cells per brain, spleen, or pair of kidneys.

Statistical analyses. Each compound was analyzed separately with data that were pooled across experiments. The experiment-to-experiment variability was accounted for by the nature of the statistical methodology performed and the inclusion of data for the control group only for those experiments in which the compound was tested. In the disseminated aspergillosis model, the 90% effective doses (ED_{90}s) were estimated by a robust probit method (22, 25) from the survival rates calculated at days 14, 21, and 28 after challenge. The technique of Kaplan and Meier (17) was used to estimate survival rates for each dose across the entire 28-day duration of the studies. Median survival times could then be determined, and the log-rank test (9) was performed to determine significant differences among compound dose groups in a pairwise manner. Adjustment for the multiplicity of these tests was made by the Bonferroni procedure. Comparisons were determined to be significant at the $\alpha = 0.05$ level. In the disseminated candidiasis model, effective ED_{50}s were estimated at days 7, 14, and 21 after challenge by the method of Knudson and Curtis (18) and are defined as the concentration of compound (in milligrams per kilogram) that protected 50% of the mice from lethal challenge. In the target organ assays, the analysis of variance test of dose-response relationships with single-degree-of-freedom contrasts was used. The mean $\log_{10}$ number of yeast CFU per organ was compared with those in the organs of sham-treated controls by the Dunnett (11) multiple comparison procedure. Inverse regression was used to estimate the doses which reduced the numbers of CFU per organ by 90% compared with those in the organs of controls.

RESULTS

Efficacy in the disseminated aspergillosis model. The ED_{90}s of L-733560, L-731373, L-705589, and AMB against a disseminated *A. fumigatus* infection in DBA/2N mice obtained on the basis of the percentage of mice treated with these compounds surviving over time are displayed in Fig. 1.

L-733560 and L-705589 significantly prolonged the survival of infected mice at concentrations of ≥0.02 and 0.08 mg/kg of body weight, respectively, compared with the survival times of infected, sham-treated animals. L-733560 levels at or greater than 0.31 mg/kg resulted in ≥85% survival at day 28 after challenge. L-705589 at levels of ≥0.08 mg/kg resulted in 95% survival at day 28. AMB at concentrations of ≥0.025 mg/kg significantly prolonged survival and gave a survival value of 80% at day 28 when it was used at 0.39 mg/kg. L-731373 was less active than L-733560 and L-705589 against *A. fumigatus*. Estimates of the ED_{90}s of L-733560, L-705589, L-731373, and AMB at day 28 after challenge were 0.48, 0.12, >20.0, and 0.36 mg/kg, respectively. ITZ was also tested and was found to be inactive in this assay (ED_{50}, >100.0 mg/kg), which may be due to the metabolism of the drug in rodents (23) or its poor solubility in AV.

Efficacy in the disseminated candidiasis mouse survival model. L-733560 administered parenterally (i.p.), was the most active of the pneumocandins against an induced disseminated *C. albicans* infection in both immunocompetent CD-1 and C′5-deficient DBA/2N mice; at day 21 after challenge ED_{50}s were 0.15 and 0.08 mg/kg, respectively (Table 1). The efficacy of L-733560 was comparable to that of i.p. administered AMB (0.30 mg/kg), and it was two- to fourfold more active than L-705589 and L-731373. The azole antifungal agents KTZ and FCZ, which were administered p.o., were active in this model and protected mice from lethal challenge with *C. albicans* (Table 1). However, ITZ was inactive (ED_{50}, >80.0 mg/kg), as was the case in the aspergillosis mouse survival model (23). L-733560 was also tested p.o. Although the ED_{50}s were measurable, efficacy was reduced ≥300-fold compared with that of L-733560 given i.p. (Table 1).

Efficacy in the disseminated candidiasis target organ assay. The pneumocandins, AMB, KTZ, and FCZ were tested for their activities in reducing recoverable yeasts from the kidneys of mice challenged i.v. with *C. albicans*. ED_{90}s were estimated on the basis of a comparison of the mean $\log_{10}$ CFU per gram of paired kidneys at day 7 after challenge in the groups treated with antifungal agents and that in sham-treated control groups. The data are given in Table 2. AMB and the pneumocandins were the most active compounds in reducing the counts in kidneys. AMB and L-733560, the most active pneumocandin, had ED_{90}s of 0.005 and 0.01 mg/kg, respectively, while L-705589 and L-731373 had ED_{90}s of 0.05 and 0.03 mg/kg, respectively. Again, as seen in the survival assay, L-733560 had activity when it was administered p.o., but the ED was considerably greater (ED_{90}, 13.3 mg/kg). FCZ was more active than KTZ in this model (ED_{90}s, 0.39 to 1.56 versus 6.4 mg/kg, respectively). Even though FCZ reduced the number of recoverable yeasts from the kidneys when it was used at concentrations of >0.39 mg/kg, it did not sterilize *C. albicans*-infected kidneys at the concentrations tested (Table 3). L-733560 and AMB sterilized the kidneys to a high degree when they were used at the higher dose levels (≥0.08 mg/kg). Even orally administered L-733560 sterilized 73% of the mouse kidneys when it was used at 50 mg/kg (Table 3). The efficacy of

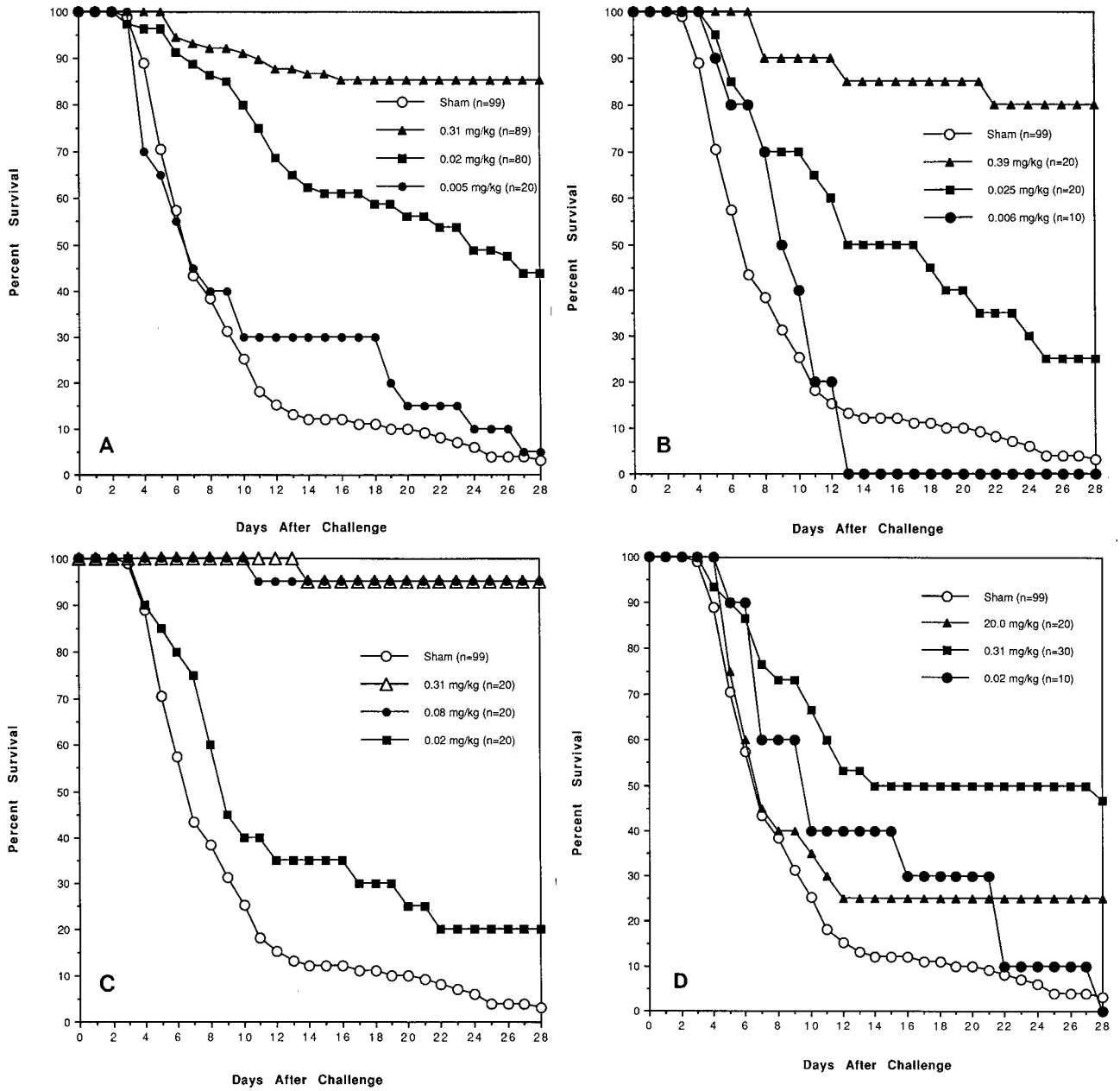

FIG. 1. Efficacy of pneumocandin analogs and AMB against a disseminated *A. fumigatus* infection (i.v. challenge with 5×10^5 to 1×10^6 conidia per mouse) in DBA/2N mice. (A) L-733560 (given i.p. b.i.d. for 5 days); 28-day ED_{90}, 0.478 mg/kg per dose (95% confidence intervals, 0.315 and 0.802). (B) AMB (given i.p. q.d. for 5 days); 28-day ED_{90}, 0.359 mg/kg per dose (95% confidence intervals, 0.223 and 0.744). (C) L-705589 (given i.p. b.i.d. for 5 days); 28-day ED_{90}, 0.122 mg/kg per dose (95% confidence intervals, 0.078 and 0.272). (D) L-731373 (given i.p. b.i.d. for 5 days); 28-day ED_{90}, >20.0 mg/kg per dose. ED_{90}s were estimated by a robust probit method for survival rates calculated at day 28 after challenge.

L-733560 over time in the TOKA is shown in Fig. 2. L-733560 rapidly cleared recoverable yeasts from the kidneys when it was used at concentrations of 0.09 and 0.375 mg/kg and the counts remained greater than 2 $\log_{10}$ CFU/g of kidney lower than those in the controls for 21 days after challenge.

Efficacy in the disseminated cryptococcosis target organ assay. At day 7 after challenge, L-733560 and AMB were tested for their activities in reducing the number of recoverable yeasts from the brains and spleens of mice infected i.v. with *C. neoformans*. L-733560 at concentrations of up to 10 mg/kg was ineffective in reducing the counts in the organs, while AMB cleared 100% of the yeasts from the organs when it was used at 0.31 mg/kg (data not shown).

DISCUSSION

The pneumocandins, especially L-733560, appear to meet many of the requirements for a new therapeutic agent including activity against clinically relevant fungal species, good aqueous solubility, favorable bioavailability, and an acceptable therapeutic index (5). L-733560, L-705589, and L-731373 have been reported to have in vitro activity against eight of the most

TABLE 1. In vivo antifungal efficacies (ED_{50}) of the pneumocandin analogs, AMB, FCZ, and KTZ in the disseminated *C. albicans* survival model[a]

Treatment	21-day ED_{50} (mg/kg) (95% confidence interval)	
	CD-1	DBA/2N
L-733560 (i.p.)	0.15 (NE)[b]	0.08 (0.04,0.15)
L-733560 (p.o.)	>50.00 (NE)	42.70 (21.7,84.2)
L-705589	0.84 (0.48,1.45)	0.62 (NE)
L-731373	0.62 (NE)	0.32 (0.17,0.67)
AMB	0.30 (0.13,0.51)	0.30 (0.13,0.51)
FCZ	9.56 (2.45,25.73)	1.03 (0.59,1.80)
KTZ	>100.0 (NE)	41.06 (22.1,76.3)

[a] ED_{50}s were estimated on the basis of survival at day 21 after challenge by using the analysis method of Knudson and Curtis (18). DBA/2N mice were infected i.v. with 10^6 cells per mouse, and CD-1 mice were infected i.v. with 10^7 cells per mouse. The pneumocandins were administered i.p. b.i.d., except for L-733560, which was also tested p.o. b.i.d. AMB was administered i.p. q.d. FCZ and KTZ were administered p.o. b.i.d. Mice received the first treatment within 15 min after challenge and were treated for a total of 4 days.

[b] NE, confidence interval could not be estimated.

clinically relevant species of *Candida*, with L-733560 being the most potent (6, 24). Growth inhibition kinetic studies against *C. albicans* demonstrated fungicidal activity, with a 99.9% reduction in growth by 3 to 5 h (6, 24). Although no evidence concerning the therapeutic potential of the pneumocandins used topically is presented here, this class of compounds has been shown to have in vivo efficacy in mouse models of oropharyngeal and gastrointestinal as well as vaginal candidiases (12, 14).

Even though the pneumocandins do not give MICs for *Aspergillus* species when they are tested by classic broth microdilution assays (6, 24), these compounds have been shown to have profound morphological effects attributed to inhibition of 1,3-β-D-glucan synthesis (19, 20). These measurable morphological effects, termed the minimal effective concentration, appear to correlate well with the potent activities of L-733560 and L-705589 in our disseminated *A. fumigatus* infection model (1). L-733560 has also been shown to be highly effective in a pulmonary aspergillosis model in rats (7). The improved efficacies of L-733560 and L-705589 appear to be the result of the substitution of an aminoethyl ether functionality at the ornithine (hemiaminal). L-731373, which lacks the aminoethyl ether group at this position, showed reduced efficacy compared with those of L-733560 and L-705589 both in vivo and in vitro in the minimal effective concentration assay.

TABLE 2. In vivo antifungal activities of pneumocandin analogs, AMB, FCZ, and KTZ against a disseminated *C. albicans* infection (TOKA)[a]

Treatment[b]	ED_{90} (mg/kg) (95% confidence interval
L-733560 (i.p.)	0.01 (0.006,0.03)
L-733560 (p.o.)	13.30 (4.70,19.20)
L-705589	0.05 (0.01,0.10)
L-731373	0.03 (0.013,0.106)
AMB	0.005 (0.001,0.10)
FCZ	0.39–1.56 (NE)[c]
KTZ	6.4 (4.9,8.0)

[a] ED_{90}s were estimated by comparison of mean $\log_{10}$ CFU per gram at day 7 after challenge for paired kidneys of treated groups with those of sham-treated controls by inverse regression analysis to determine which doses reduced the counts in the organs by 90%. DBA/2N mice were infected i.v. with 7.4×10^4 cells per mouse.

[b] The pneumocandins were administered i.p. b.i.d., but L-733560 was also tested p.o. b.i.d. AMB was administered i.p. q.d. FCZ and KTZ were administered p.o. b.i.d. Mice received the first treatment within 15 min after challenge and were treated for a total of 4 days.

[c] NE, confidence interval could not be estimated.

TABLE 3. In vivo antifungal efficacies of L-733560, AMB, and FCZ against a disseminated *C. albicans* infection (TOKA)[a]

Drug and treatment group (dose [mg/kg])	Mean $\log_{10}$ CFU/g	% Sterilization	Sample size
L-733560 (i.p.)			
Sham treated	6.44	0	78
0.375	2.19[b]	100.0	65
0.09	2.45[b]	62.5	80
0.045	3.39[b]	15.0	20
0.023	4.92[b]	1.4	74
0.005	6.25	0	22
AMB (i.p.)			
Sham treated	6.44	0	10
0.31	2.33[b]	60.0	10
0.08	2.35[b]	40.0	10
0.02	4.30[b]	0	10
0.005	5.68	0	8
FCZ (p.o.)			
Sham treated	7.08	0	10
25.0	4.75[b]	0	10
6.25	4.67[b]	0	10
1.56	4.22[b]	0	10
0.39	3.92[b]	0	10
0.10	6.49	0	6
L-733565 (p.o.)			
Sham treated	5.84	0	15
50.0	2.23[b]	73.0	15
25.0	2.93[b]	30.0	10
12.5	4.41[b]	0	10

[a] Mean $\log_{10}$ CFU per gram at day 7 after challenge for paired kidneys. Percent sterilization indicates number of mice with no detectable yeasts; the limit of detection was 50 yeast cells per pair of kidneys. DBA/2N mice were infected i.v. with 7.4×10^4 cells per mouse. L-733560 was administered b.i.d. either i.p. or p.o. AMB was administered i.p. q.d., and FCZ was administered p.o. b.i.d. Mice received the first treatment within 15 min after challenge and were treated for a total of 4 days.

[b] Significant reduction compared with that in the sham-treated controls ($\alpha = 0.05$).

L-733560 exhibited weak but measurable in vitro activity (minimum fungicidal concentrations, 16 to 32 μg/ml) against clinical isolates of *C. neoformans* (6, 24), which did not translate to efficacy in our in vivo model.

L-733560 administered both parenterally and orally has been shown to exhibit potent in vivo activity against *P. carinii* cysts in an immunocompromised rat model (26). Other lipopeptides have also been shown to have anti-*Pneumocystis* activity (3, 10, 26, 27).

Preliminary mouse toxicity tests with the pneumocandins administered as a single i.v. bolus have shown favorable therapeutic indices (ratio of maximum tolerated dose to efficacy in a disseminated candidiasis survival model) (5). Comparative pharmacokinetics with pneumocandins in mice and rhesus monkeys have shown good bioavailability. L-733560 had half-lives of 5.95 and 11.8 h in mice and rhesus monkeys, respectively, (15).

The potent activities of the water-soluble pneumocandins L-705589, L-731373, and L-733560 in murine models against *A. fumigatus*, *C. albicans*, and *P. carinii* combined with a low toxic

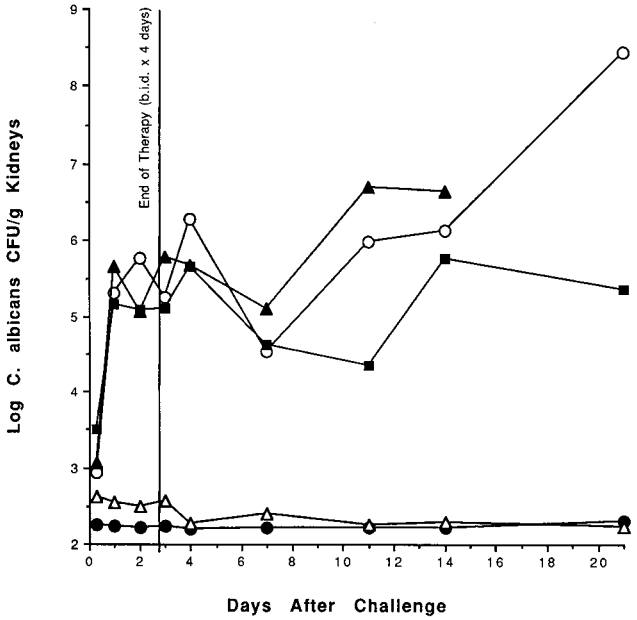

FIG. 2. Efficacy of L-733560 against a disseminated *C. albicans* infection in DBA/2N mice (TOKA). ○, sham-treated animals; ▲, 0.005 mg/kg (0% sterilization); ■, 0.023 mg/kg (40% sterilization); ●, 0.09 mg/kg (100% sterilization); △, 0.375 mg/kg (100% sterilization).

potential and good bioavailability make the pneumocandins potentially attractive candidates for use in the treatment of opportunistic and secondary infections in immunocompromised patients.

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